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Attraction of female *Culex quinquefasciatus* Say (Diptera: Culicidae) to odors from chicken feces

Miriam F. Cooperband [a,b,*], J. Steven McElfresh [a], Jocelyn G. Millar [a], Ring T. Cardé [a]

[a] Department of Entomology, University of California, Riverside, CA 92521, USA
[b] Center for Medical, Agricultural, and Veterinary Entomology, USDA-ARS, Gainesville, FL 32608, USA

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ABSTRACT

Odors from fresh chicken feces in water elicited upwind flight of host-seeking female *Culex quinquefasciatus* mosquitoes in a dual-choice olfactometer. Acidification of the slurry of chicken feces and water resulted in increased attraction, whereas alkaline slurries of chicken feces and water controls did not attract female mosquitoes. This is the first reported example of avian fecal odor eliciting upwind flight of female mosquitoes. Headspace odors from acidified slurries were sampled using solid phase micro-extraction (SPME) coated fibers. Eight volatile aldehydes [(*E*)-2-decenal, undecanal, dodecanal, tetradecanal, pentadecanal, hexadecanal, heptadecanal, and octadecanal] identified in the headspace of acidified chicken feces elicited electroantennogram responses from antennae of *C. quinquefasciatus* females. An improved electroantennogram technique in which four antennae were used in parallel for monitoring the GC effluent is described.

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1. Introduction

Mosquitoes that feed on birds, such as *Culex tarsalis* and *Culex quinquefasciatus*, are important vectors of West Nile virus, Saint Louis encephalitis, Western Equine encephalitis, and Japanese encephalitis (reviewed in Lehane, 1991; Goddard et al., 2002). Female mosquitoes locate and select hosts for a blood meal using a range of odor, visual, and thermal cues from their hosts (reviewed in Clements, 1999). In principle, host finding begins by location of the host habitat (Vinson, 1985). Host-seeking, ornithophagous mosquitoes, such as *C. tarsalis* (Reeves et al., 1963), have been reported to fly mostly around elevated vegetation and ecotones (Lothrop and Reisen, 2001; Lothrop et al., 2002), the typical habitat of the majority of nesting and roosting birds (Gates and Gydel, 1978). It is thought that visual cues associated with the landscape's features contribute to mosquitoes locating this habitat (Lothrop and Reisen, 2001). Once in the host habitat, a more specific response to host odors can occur. Close to a host, visual and thermal cues also may be employed in the final steps of host location (Takken, 1991). Detection of CO_2, the first known mosquito attractant (Rudolfs, 1922; Gillies, 1980; Clements, 1999), activates host-seeking females of many mosquito species, and elicits upwind flight (Dekker et al., 2005). Carbon dioxide may be especially important in attraction of generalist feeders, such as *Anopheles quadriannulatus*, whereas cues that are more host-specific may play a greater role in mosquitoes with a limited range of hosts (Mboera and Takken, 1997; Takken and Knols, 1999; Dekker et al., 2001a,b, 2002).

In North America, the primary hosts of *C. quinquefasciatus* and *C. tarsalis* are birds (Dow et al., 1957; Reeves et al., 1963; Bohart and Washino, 1978; Reisen et al., 2002). Early research on *C. tarsalis* showed that attraction and biting frequency differed with host species (Dow et al., 1957; Reeves et al., 1963). However, specific host odors, the rate of CO_2 production, and levels of defensive behaviors vary with different hosts. All of these factors can contribute to differences in attraction and biting (Reeves et al., 1963; Anderson and Brust, 1996). Other host odors to which hematophagous mosquitoes respond include odors associated with human skin, sweat, and breath, and odors from cattle, birds, and mice (reviews, Clements, 1999; Takken and Knols, 1999). Other than CO_2, specific compounds that have been found to be "attractive" using various assays include acetone (Takken et al., 1997), 1-octen-3-ol (Takken and Kline, 1989), fatty acids (Knols et al., 1997; Bosch et al., 2000), and from human skin, L-lactic acid (Acree et al., 1968; Geier et al., 1996) as well as carboxylic acids, alcohols, and aldehydes (Puri et al., 2006; review, Bernier et al., 2006). Table 1 lists host odors and their sources that have been determined to be attractive to mosquitoes without the addition of CO_2.

Synergistic and additive effects also may occur. Blends of 1-octen-3-ol with CO_2 were reported to be more attractive than CO_2 alone for some mainly ornithophagous mosquitoes such as

* Corresponding author. Current address: USDA-APHIS-PPQ, Otis ANGB, Building 1398, West Truck Road, MA 02542, USA. Tel.: +1 508 563 9303x266.
E-mail address: Miriam.F.Cooperband@aphis.usda.gov (M.F. Cooperband).

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Table 1
Specific odors that elicit attraction of female host-seeking mosquitoes without supplemental CO_2, and the genus in which they occur

Mosquito genus	Attractant	Source	Reference
Aedes, Anopheles	L-Lactic acid	Human	Acree et al. (1968), Dekker et al. (2002)
Aedes, Anopheles, Coquillettidia, Mansonia	1-Octen-3-ol	Bovine	Takken and Kline (1989), Kline et al. (1990)
Aedes, Anopheles	Short chain fatty acids	Limburger cheese, Human	Knols et al. (1997), Bosch et al. (2000)
Aedes, Anopheles	Ammonia	Human	Geier et al. (1999), Braks et al. (2001), Smallegange et al. (2005)
Aedes	L-Lactic acid blended with dichloromethane, dimethyl sulfide, or acetone	Human, other	Bernier et al. (2003)
Culex	Carboxylic acids, alcohols, and aldehydes	Human	Puri et al. (2006)

Culex salinarius (Kline, 1994; Kline and Mann, 1998; Rueda et al., 2001). However, in other primarily ornithophagous species, such as *Culex nigripalpus*, *Culex pipiens*, *C. quinquefasciatus*, and *C. tarsalis*, blends of CO_2 with 1-octen-3-ol did not result in increased attraction compared to use of CO_2 alone (Takken and Kline, 1989; Kline et al., 1991; Kemme et al., 1993; Kline, 1994; Burkett et al., 2001; Rueda et al., 2001), and in some cases the binary combination was actually less attractive than CO_2 alone (Mboera et al., 2000; Burkett et al., 2001; Russell, 2004). Furthermore, 1-octen-3-ol has not been reported as an odor associated with birds. Odors from human skin (Puri et al., 2006), bird feathers (Allan et al., 2006), and bird uropygial glands (Russell and Hunter, 2005) also have been identified as potential sources of attractants for *Culex* spp. To our knowledge, no specific compounds attractive to host-seeking mosquitoes have been identified from avian sources.

We became interested in the role of bird droppings as a source of host-location cues based on observations that starved *Culex* mosquitoes probed chicken feces with their mouthparts (MFC, unpublished). In a preliminary trial, we used a dual-choice olfactometer to test for attraction to odors from chicken feces (without added CO_2) using mated *C. quinquefasciatus* females with no previous blood-feeding experience, and deprived of sugar and water overnight. We found that within 1 min, mosquitoes flew to the bird feces odor rather than to the control 74% of the time. This report documents the bioassays conducted to examine the attraction of *C. quinquefasciatus* to odors released from chicken feces, our process of odor-collection, and the identification and bioassay of the constituents that attract *C. quinquefasciatus*, using gas chromatography (GC), mass spectrometry (MS), and coupled GC-electroantennogram (EAG) assays.

2. Materials and methods

2.1. Insects

Fourth instar larvae of *C. quinquefasciatus* were obtained from William Walton (Department of Entomology, UC Riverside), and reared under a 16:8 h L/D photoperiod, at ~26 °C. The colony has been in culture since 1991 (Georghiou and Wirth, 1997) and was reared on a larval diet consisting of one part brewer's yeast to three parts ground mouse chow. Colony adults were blood fed upon live 1-d-old *Gallus gallus domesticus* chicks (Animal Use Protocol #A-0303007-1). Pupae were collected daily and allowed to emerge in 30 cm L × 30 cm W × 30 cm H screened cages. Two to 5 d prior to testing, a cage containing 100–200 adult male and female mosquitoes was transferred into a light box with a reversed daylight cycle of 16:8 h L/D (with an hour of decreased light during dusk and dawn) and maintained at ~26 °C and 50–60% RH. Adults were provided with 25% honey–water solution until 12 h prior to experiments. Female mosquitoes were about 2-week-old when used in experiments, and had no prior host-feeding experience.

2.2. Bioassays

Bioassays were conducted using a dual-choice olfactometer designed to measure upwind attraction, similar to those described in Geier et al. (1999) and Bosch et al. (2000) (Fig. 1). Laboratory air was forced through an activated charcoal filter, a Y-tube splitter, into two 50-ml Erlenmeyer flasks and out the side arms through Tygon tubing (replaced each experimental run), and into the two arms of the dual-choice olfactometer. The most upwind section of the olfactometer consisted of two 45-cm lengths of black PVC pipe (diameter 10.5 cm). Air flowed through each of the two black cylinders, through nylon mesh screens (to prevent mosquito entry), and into Plexiglas® cylinders (diameter 10 cm, 35 cm long), each containing a rotatable mesh valve which could be closed at the conclusion of experiments. The two cylinders were attached to a Plexiglas® box (11 cm H × 30 cm W × 21 cm L) opposite a single Plexiglas® cylinder (10 cm diameter, 42 cm long) where mosquitoes were individually released from release cages. Release cages were Plexiglas® cylinders (7 cm diameter, 5 cm long) with nylon mesh covering one end and a slit cut near the other end allowing a clear acetate cover to be slid in and out (Fig. 1b). Airflow was visualized by inserting a pipette tip with $TiCl_4$ into the upwind end of the choice arms of the olfactometer. By video recording and digitizing the downwind movement of the $TiCl_4$ smoke through the entire bioassay apparatus, using the motion analysis software Motus (Peak Performance Technologies, Inc., Centennial, CO, USA), airspeed was determined to be 10 cm s^{-1}. The current of air flow was linear until it reached the downwind wall of the central chamber, at which point it made a sharp turn toward the central entry tube and then another sharp turn into that tube, after which it continued straight and out of the system (see dashed arrows in Fig. 1). Air adjacent to the upwind wall of the central chamber, between the two choice tubes, was relatively motionless. All bioassays were video recorded using a Sony Hi8 (EVO-550H) recorder and Sanyo black and white video cameras (VCB-3512T, with shutter speed of 1/60 s) with a 6 mm lens. An infrared light (940 nm) was placed approximately 30 cm above the downwind end of the chamber for illumination of the video recording. A 20-cm television monitor located behind a black curtain was used to observe mosquitoes during the experiment and to provide a low amount of indirect visible light. Between bioassays, the apparatus was dismantled, washed with soap and warm water, rinsed three times with deionized water, and allowed to dry.

In a dimly lit room, female mosquitoes were aspirated individually into release cages 20–22 h prior to experiments (just before their dawn). The cages were placed on trays in a light box (also with a reversed photophase of 16:8 h L/D) in the room with the dual choice olfactometer. Experiments were conducted 2–4 h following the artificial dusk, when feeding typically occurs (Meyer et al., 1985, 1986). Experiments took place in the dark except for the light from the television monitor and an Infrared LED Illuminator with an array

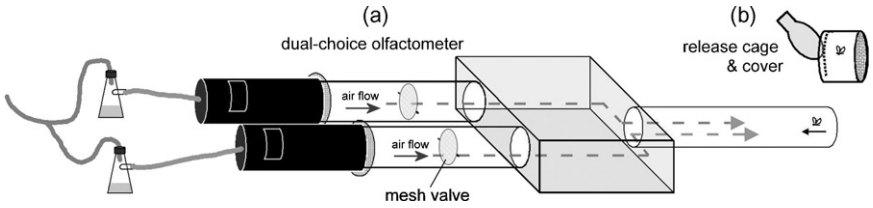

Fig. 1. Diagram of the dual choice olfactometer (a). Air flowed from left to right and individual mosquitoes were released at the right when a cover was removed from a release cage (b). Test compounds were placed in aqueous solutions in the flasks on the left. After entering the rectangular central chamber, a choice was made when the mosquito entered one of the upwind tubes.

of 90 LEDs and a peak output of 880 nm (Tracksys Ltd., Nottingham, UK). Between releases, if additional light was needed a small flashlight with a red filter (Rosco #27) was used. A release cage containing a single mosquito, with the acetate cover in place, was placed against the entry end of the dual-choice olfactometer. The cover was removed, allowing the mosquito to fly into the dual choice olfactometer.

Behavioral observation commenced at the time of mosquito release. Each mosquito was observed for 1 min for the following behaviors: activation (mosquito took flight), upwind orientation (mosquito reached the central chamber), and choice (mosquito arrived in one of the two upwind arms).

2.3. Testing odor choices

Bioassays were conducted to test the activity of the following odor sources:

(a) *fresh chick feces*: aqueous suspensions of fresh droppings from 3-d-old chicks, collected on paper towel;
(b) *aged chicken feces*: field-collected droppings of unknown age collected from old dry manure mounds that had dried after caged adult chickens had been removed;
(c) *chicken feces*: field-collected droppings of unknown age, collected from fresh manure mounds beneath caged adult chickens, and included moist, fresh droppings;

and solutions of droppings in (c) with:

- pH adjusted to approximately 2.0 (range 2.0–2.5);
- pH adjusted to 11.0; or
- pH left unadjusted at 7.3.

To collect fresh chick feces, chicks were placed individually on a square of paper towel (7.5 cm × 10 cm) in a hardware cloth cage (6.5 cm × 8.5 cm × 9 cm, mesh of 0.6 cm) for 4 h. Generally, during this time a chick produced one dropping. The paper towel with the dropping was removed and frozen until needed. For the bioassay, a paper towel square with a chick dropping was placed in one Erlenmeyer flask, a clean square (control) was placed in the other flask, 20 ml of distilled water were added to each, and the flasks were connected to the bioassay setup as described above. The experiment was repeated using 10 g of adult chicken droppings from dry manure mounds, and fresh moist mounds beneath outdoor chicken cages, each mixed with 20 ml water, and compared to a neutral control of 20 ml of water. The pH of fresh adult chicken droppings in water was either left unmodified (pH 7.3), brought to 2.0 (range 2.0–2.5) by adding phosphoric acid, or brought to 11.0 using sodium hydroxide solution. These three mixtures were then compared against each other in bioassays (positive controls). As a control, water samples brought to pH 2.0 or pH 11.0 were also compared in the bioassay.

To examine the possibility of attraction due to ammonia, a known emanation from chicken feces (Witter, 1991), female mosquitoes were tested against ammonia at four different concentrations in water (1, 10, 100, and 1000 ppm). In the ammonia tests, 20 mosquitoes were released simultaneously in the olfactometer. This was repeated twice for each treatment.

2.4. Fractionation and testing of active slurry

Chicken droppings were collected from fresh piles beneath chicken cages, and 350 g of chicken droppings were added to 1050 ml of distilled water. The pH was adjusted to 2.0–2.5 with phosphoric acid, chosen because it is nonvolatile. The mixture was poured into a 5-l round-bottomed boiling flask fitted with a still head and condenser, and heated to reflux. Four 150-ml fractions of distillate were collected and divided into aliquots, which were serially diluted and frozen until needed, and the residue was discarded. Each of these fractions was tested at five concentrations in water (equivalent to 0.7 g ml^{-1}, 0.1 g ml^{-1}, 0.01 g ml^{-1}, 0.001 g ml^{-1}, and 0.0001 g ml^{-1}) individually in the bioassay versus water controls. They were also recombined and tested at two concentrations (0.001 g ml^{-1} and 0.0001 g ml^{-1}) in the bioassay compared to water. Because the residue had been discarded, the distillation was conducted a second time in the same manner, except that all of the distillate was collected in one fraction and the residue was retained. These two fractions were tested in the bioassay at 0.1 g/ml compared to water.

2.5. Preparation of homogeneous aliquots

Aluminum foil was placed beneath cages of adult chickens housed at UC Riverside (fed on 17% lay mash, Kruse's Perfection Brand, Goshen, CA). After 2 d, the foil was collected and about 5 kg chicken droppings were homogenized in a food processor. Homogenized droppings were then divided into 10 g aliquots and frozen in 40 ml glass vials with Teflon-lined lids. Prior to use, a vial with 10 g of homogenized chicken droppings was defrosted and the contents were mixed with 10 ml distilled water and 1.2 ml phosphoric acid to bring the mixture to approximately pH 2.0.

2.6. Solid-phase micro-extraction (SPME)

Headspace volatiles for use in GC-EAD were collected using an aliquot of chicken dropping solution (prepared as described above) placed in a 100-ml beaker and covered with aluminum foil. A 100-μm polydimethylsiloxane-coated SPME fiber (Supelco, Bellefonte, PA) was inserted through the foil and exposed in the headspace. Initially, headspace volatiles were collected for various amounts of time (0.5, 1, 2, 4, 24, 48, 72, and 96 h), each resulting in a similar pattern of GC peaks, with peak size increasing as collection time increased. Therefore, SPME fibers were exposed to headspace volatiles for a period of 4 d in order to collect enough material to produce strong antennal responses.

2.7. Collection of volatiles on activated charcoal

Headspace volatiles for use in GC–MS were collected from a 40 ml vial on activated charcoal from aliquots of acidified (pH 2) aqueous homogenized chicken dropping mixture. Thus, 10 ml of the acidified slurry were placed in a 40 ml vial with a cap fitted with an air inlet and the outlet fitted with a charcoal trap consisting of a pipette containing 0.36 g activated charcoal (50–200 mesh, thermally desorbed, held in place by small plugs of glass wool). Medical air was piped through the apparatus at 20 ml min^{-1}. Odors were collected for 4 d, after which the charcoal trap was rinsed three times with 1 ml of dichloromethane. The combined dichloromethane extracts were stored at −7 °C until needed.

2.8. Electroantennograms

Mosquitoes were prepared for experiments as described above. Adult female *C. quinquefasciatus* mosquitoes, about 2-week-old (as in Cooperband and Cardé, 2006), were used for electroantennograms. Two or more days prior to an experiment, a cage containing adult males and females with no prior host-feeding experience was placed in the reversed photoperiod light/dark box with 16:8 h L/D (with 1 hr of reduced light to simulate dusk and dawn). Mosquitoes were deprived of honey solution and water for 20–22 h prior to testing, and were used 1–3 h after their dusk. A cover made of washed Duvetyne black fabric was used to keep caged mosquitoes in the dark, and the lights in the lab were kept off during the preparation and use of mosquito antennae. The cage sleeve was pulled out through a slit in the black fabric cover and the experimenter's breath was used to attract host-seeking females. A female that flew toward the experimenter's breath was collected from the sleeve using forceps.

Under a dissecting microscope, on a piece of filter paper moistened with saline (7.5 g/l NaCl, 0.21 g/l $CaCl_2$, 0.35 g/l KCl, and 0.2 g/l $NaHCO_3$), the female mosquito was decapitated with a razor blade. The tip of the last antennal segment was sliced off each antenna and the antennae were sliced from the head basal to the Johnston's organ. The antennae including the Johnston's organ then were placed between two glass capillary tubes containing the saline and gold wire electrodes. In total, four antennae were placed in parallel between the capillaries, with the four distal tips in the end of one, and the four proximal ends in the other. Typically, the four antennae were taken from two individuals. Studies with moths have found that using multiple antennae in parallel could improve the signal-to-noise ratio (Moore, 1981; Park and Baker, 2002).

The electrodes were connected to a custom-made amplifier (as in McElfresh et al., 2001) and secured to a stand equipped with micromanipulators. The antennal preparation was maneuvered into the opening of an L-shaped glass stimulus delivery tube (1.5 cm diameter). The entire apparatus was inside a Faraday cage, and the antennal preparation was shielded with a smaller Faraday cage. Medical air was passed through a glass wool pad moistened with distilled water, into the stimulus delivery tube, and over the antennae at 0.5–0.8 l min^{-1}. Effluent from the GC column was split (see below), with half going to the stimulus delivery tube via a heated outlet port (250 °C). The activity of each antennal preparation was tested with a mixture of four synthetic standards known to elicit antennal responses from *C. quinquefasciatus* females that were either gravid (Du and Millar, 1999) or host seeking (Puri et al., 2006). The fifth compound, 1-octanol, was included in the mix as an internal standard. This control mixture contained 100 ng μl^{-1} each of nonanal, 4-ethylphenol, indole, 2-tridecanone, and 1-octanol in dichloromethane, and 1.0 μl aliquots of this mixture were injected (i.e., 50 ng of each compound passing over the antennal preparation). Each antennal preparation was used twice. In cases where the antennae did not respond to the five known compounds, the preceding test of unknowns was discarded.

2.9. Gas chromatography and mass spectrometry

The EAG was interfaced to a Hewlett-Packard 5890 Series II GC equipped with a DB-5 column (30 m × 0.25 mm i.d. × 0.25 μm film, 2 m retention gap of 0.25 mm i.d. deactivated fused silica column; J & W Scientific, Folsom, CA), using splitless injection with He carrier gas. The column effluent was split between two 0.32 mm ID branches with a fused silica X-cross, with the remaining arm of the cross supplying He makeup gas (5 ml/min). One branch was directed to the flame ionization detector (FID), and the other to the EAG. Data from the GC-EAG were recorded at 5 Hz using a PeakSimple Chromatography Data System and PeakSimple software (version 3.21, SRI Instruments Inc., Torrance, CA), with readings zeroed at the start of each run. The temperature program for the GC oven consisted of an initial 1 min hold at 40 °C, then ramped at 10 °C/min to 250 °C, and was held at that temperature for 30 min.

Extracts of acidified feces were analyzed with an HP 6890 GC in splitless mode interfaced to a 5973 mass selective detector with electron impact ionization (70 eV). A 30 m × 0.25 mm i.d. HP-5MS column was used with the temperature program described above. Retention indices for the two GCs were calculated to match peaks from the GC-EAG and GC–MS instruments, and compounds were identified by comparison of mass spectra and retention times with those of authentic standards.

2.10. Identification and testing of active components

Synthetic chemicals were examined for their ability to elicit antennal responses. Pentadecanal, hexadecanal, heptadecanal, and octadecanal, which were not commercially available, were synthesized by Swern oxidation of the corresponding alcohols (from Aldrich Chemical Co., Milwaukee, WI) (Mancuso et al., 1978). All other compounds were obtained from Aldrich Chemical Co. (Milwaukee, WI), except for 1-octanol (TCI America, Portland, OR) and undecanal (Alfa Aesar, Ward Hill, MA). Synthetic chemicals were diluted to 100 ng μl^{-1} and combined in heptane. Antennal responses to synthetic compounds were tested using the GC-EAG setup as described above.

2.11. Statistical analyses

Bioassays involving mosquitoes in the dual-choice olfactometer were analyzed using a χ^2 goodness-of-fit test ($P < 0.05$) to test the null hypothesis that each port was chosen at the same frequency (Sokal and Rohlf, 1995).

3. Results

3.1. Behavioral responses to odors

Odor comparisons in the olfactometer, numbers of mosquitoes tested, and the choices made by host-seeking female mosquitoes are presented in Fig. 2. Control olfactometer tests of wet paper towel versus wet paper towel resulted in low activation (28.6%) and choices were not significantly different, indicating no directional bias in the system. Most odor comparisons in Fig. 2 revealed no significant preference by mosquitoes for one test stimulus over the other. Exceptions were for fresh chick feces on paper towel in water which was chosen more often (62%) than paper towel in water (38%), and pH 2.0 chicken feces in water

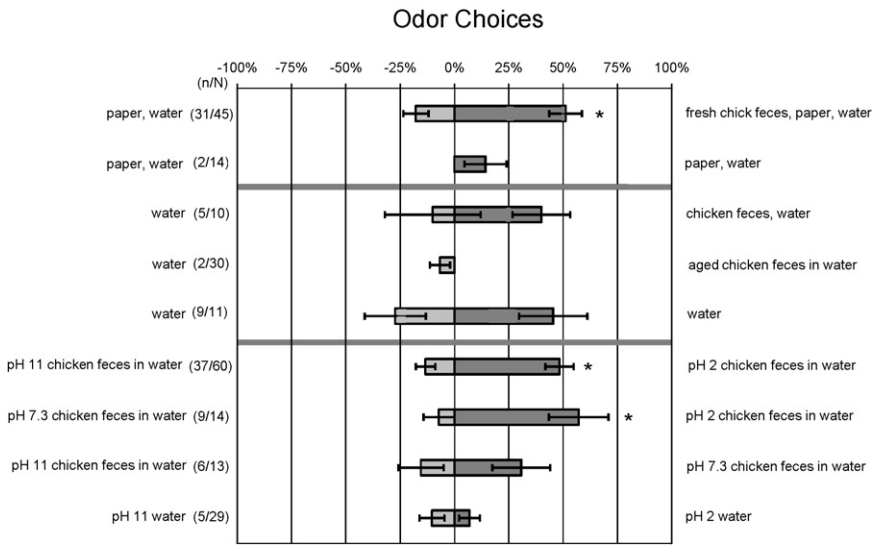

Fig. 2. Choices of mosquitoes in a dual-choice olfactometer when offered various odors. The results are expressed as percentage of the number of mosquitoes released that entered the control port (light grey) or test odor port (dark grey) (±standard error). The length of each bar represents the percentage of mosquitoes tested that made a choice. The numbers *n* and *N* indicate the number of mosquitoes that made a choice and the number of mosquitoes tested, respectively. The difference between them represents the number of mosquitoes tested that did not orient upwind and enter a port. Asterisks denote significance using a χ^2 goodness-of-fit test with $P < 0.05$, indicating that the two sides were not chosen at the same frequency.

which was chosen more often (67%) than pH 11.0 chicken feces (33%), and also chosen more often (62%) than pH 7.3 chicken feces (38%) (Fig. 2). The number of mosquitoes that chose chicken feces in water at pH 7.3 did not differ from the number that chose feces in water at pH 11.0, and the number that chose water at pH 2.0 did not differ from the number that chose water at pH 11.0. Dried chicken feces in water resulted in low levels of activation (37%, $N = 30$) and there was no preference for the aqueous suspension of dried chicken feces compared to water, suggesting that the attractive compounds present in fresher chicken feces were volatile and/or unstable, and were no longer present. Mosquitoes chose fresh chicken feces suspensions which had been acidified significantly more often than unadjusted neutral or alkaline mixtures, suggesting that the attractive compounds were acidic, or were released from precursors under acidic conditions.

3.2. Behavioral responses to ammonia

When 40 mosquitoes were tested with ammonia concentrations of 1, 10, 100, and 1000 ppm, upwind orientation rates were 50%, 60%, 100%, and 98%, respectively. There were no significant differences in attractiveness between any of the concentrations of ammonia tested and the water controls, except at the highest concentration, in which significantly more mosquitoes (15 of the 19 mosquitoes that made a choice) selected the control port (χ^2 goodness-of-fit test, $P < 0.05$).

3.3. Behavioral responses to slurry fractions

The four fractions of distillate obtained from acidified chicken feces were tested in the dual-choice olfactometer, each at five different concentrations. At the highest concentration (0.7 g ml^{-1}) few mosquitoes oriented upwind and most mosquitoes remained in their release cages or descended to the floor of the entry tube after activation. None of the fractions were chosen over the water controls at any concentration tested. When all four fractions were recombined and tested at 0.0001 and 0.001 g ml^{-1}, rates of upwind orientation were 6.7 and 20%, respectively ($N = 15$) and there were no significant differences between test odors and controls. Similarly, the second set of two fractions of acidified chicken feces (the distillate ($N = 15$) and the residue ($N = 13$)), tested at 0.1 g ml^{-1} each, gave upwind orientation rates of 33 and 46% respectively, but choices did not significantly differ from the water controls.

3.4. GC-EAG analyses and identification of bioactive compounds

Representative GC-EAG chromatograms of the headspace volatiles of aqueous slurries of unaltered chicken feces and acidified chicken feces in water are shown in Fig. 3. Acidification of chicken feces resulted in a drastic change in the odor profile of the slurry, mirrored by changes in the profile of antennal responses elicited by the various compounds in the acidified extract. Eighteen compounds from the acidified chicken feces sample elicited antennal responses (Fig. 3b). Peaks 2, 4, 5, 6, 7, 8, 11, 14, 16, and 18 were identified by GC–MS and confirmed with standards

Table 2
Synthetic chemicals tested in coupled gas chromatography-electroantennogram analyses, using *Culex quinquefasciatus* mosquitoes

Active peak no. in headspace of acidified chicken feces	Compound	Solvent
	1-Octanol[a]	Dichloromethane
2	Nonanal[a,b,c]	Dichloromethane
4	4-Ethylphenol[a]	Dichloromethane
	Indole[a]	Dichloromethane
5	(*E*)-2-Decenal[c]	Heptane
6	Undecanal[c]	Heptane
7	Dodecanal[b,c]	Heptane
	2-Tridecanone[a]	Dichloromethane
8	Tetradecanal[c]	Heptane
11	Pentadecanal	Heptane
14	Hexadecanal	Heptane
16	Heptadecanal	Heptane
18	Octadecanal	Heptane

[a] Compounds used in control blend.
[b] Found in human skin odor (Bernier et al., 2000).
[c] Found in human skin odor (Curran et al., 2007), however, their identification of tetradecanal was tentative and probably incorrect because its reported retention time was less than that of tridecanal confirmed with a standard.

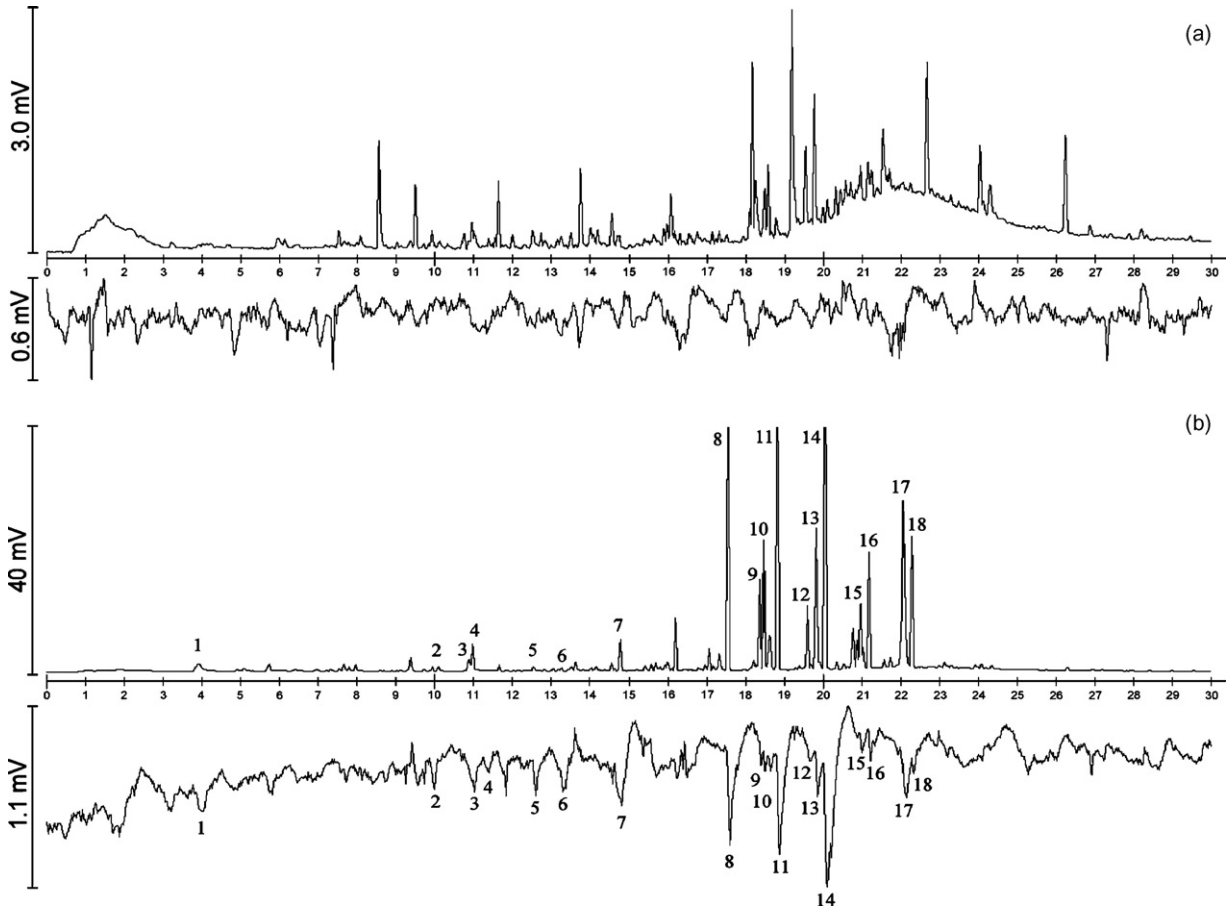

Fig. 3. GC profile and corresponding antennal responses using quadruple antennal preparations from female *Culex quinquefasciatus* mosquitoes for (a) headspace odors from chicken feces mixed with water (collected on SPME fiber over 3 d) and (b) headspace odors from chicken feces mixed with water and acidified to pH 2 (collected on SPME fiber over 3.8 d). GC traces are shown above inverted EAG traces. Numbers indicate GC peaks to which an EAG response was reproducibly observed (GC peaks 8, 11, and 14 are truncated). Identification of peaks 2, 4–8, 11, 14, 16, and 18 are provided in Table 2. The remaining 8 peaks have not been identified. Tick marks on the *x*-axis indicate elapsed minutes.

(see Table 2). Peaks 2 and 4 were identified as nonanal and 4-ethylphenol, which were also compounds used in the control blend. Synthetic standards of the eight other identified compounds elicited antennal responses from females when tested by GC-EAD (Fig. 4). Comparisons of the EAG responses elicited from single versus quadruple preparations of antennae from females showed that four antennae in parallel produced both higher amplitude and more consistent signals (Fig. 5).

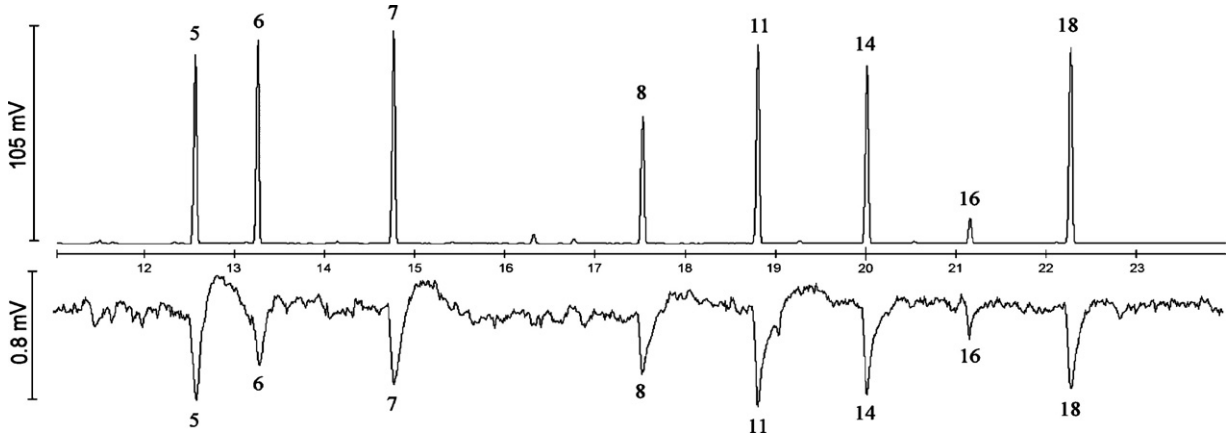

Fig. 4. A single GC trace (top) and inverted EAG trace (bottom) of eight synthetic components that were found in headspace volatiles of acidified chicken feces in water. The EAG shows the response of a quadruple antennal preparation from female *C. quinquefasciatus* mosquitoes. Components were tested at 50 ng each except for #16 which was injected at 5 ng, as follows: (5) (*E*)-2-decenal, (6) undecanal, (7) dodecanal, (8) tetradecanal, (11) pentadecanal, (14) hexadecanal, (16) heptadecanal and (18) octadecanal. Tick marks on the *x*-axis indicate elapsed minutes.

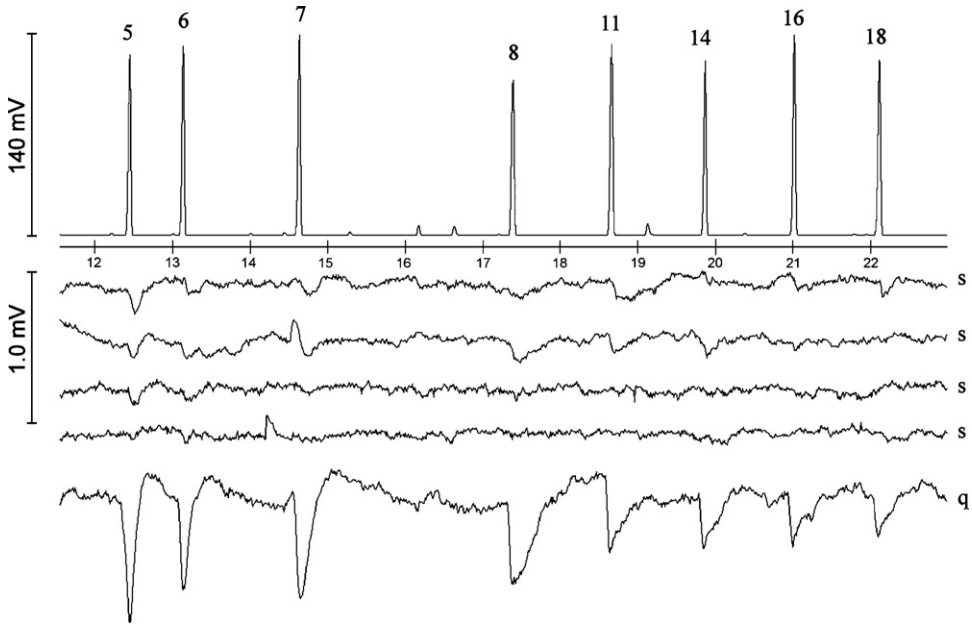

Fig. 5. A single GC trace (top) and five inverted EAG traces (bottom) from antennae of female *C. quinquefasciatus* mosquitoes responding to eight synthetic components that were found in acidified chicken feces in water. The EAG traces show responses of four single antennal preparations (s), and one quadruple antennal preparation (q). Components were injected at 50 ng each as follows: (5) (*E*)-2-decenal, (6) undecanal, (7) dodecanal, (8) tetradecanal, (11) pentadecanal, (14) hexadecanal, (16) heptadecanal and (18) octadecanal. Tick marks on the *x*-axis indicate elapsed minutes.

4. Discussion

Crepuscular ornithophagous species such as *C. quinquefasciatus* and *C. tarsalis* must locate relatively small, inconspicuous hosts roosting in trees in low light levels, and chemical cues play a key role in host location. However, relatively little is understood about the actual roles of specific chemicals, and few chemicals have been identified and found to elicit upwind attraction in host-seeking mosquitoes. Complicating our understanding, studies examining mosquito "attraction" have used various types of bioassays, some involving movement toward an odor source in still air and others monitoring upwind displacement when in the odor plume. Our bioassay measured the proportion of mosquitoes that flew upwind into the central chamber and chose the stimulus port within 1 min of release. Rapid responses by mosquitoes in the correct physiological state are more likely to indicate attraction than summed responses over many minutes, because the accumulation of mosquitoes in a choice assay can increase with time (Dekker et al., 2001b). A limitation of the dual-choice olfactometer is that mosquitoes only have two choices, and so the olfactometer only measures whether one stimulus is preferred over another stimulus, or over a control. The use of a positive control, rather than a neutral control stimulus in choice assays may be helpful in establishing attraction (Cardé, 2005).

In the work reported here, we showed that the ornithophagous mosquito, *C. quinquefasciatus* in North America, oriented upwind to odors from host feces, suggesting that this response may be used to locate the host's habitat. We believe that this is the first definitive documentation of host feces being attractive to mosquitoes. Fresh (1–2-d old) chick and chicken feces were attractive to mosquitoes, whereas older, dried chicken feces were not attractive, suggesting that the attractive components are volatile compounds emanating from fresh chicken feces. If host-seeking mosquitoes use host feces to locate host habitat, they may benefit less or not at all from attraction to old host feces, whereas fresh feces are indicative of more recent host presence. Support for the idea that host feces may play a role in attracting mosquitoes to their hosts was found in a field study by Becker et al. (1995), in which the attraction of mosquitoes to CO_2 versus to a caged hamster was compared. It was noted that at first, mosquitoes (including *C. pipiens*) were more attracted to the CO_2, but on the third and fourth days, after waste had accumulated in the cage, more mosquitoes were attracted to the hamster cage than to the CO_2 source, but no follow up studies were done to confirm the source of the attractants. Field trapping rates of the sand fly *Phlebotomus papatasi* (Diptera: Psychodidae) improved when traps were baited with year-old cattle manure, but this attraction may have been related to finding oviposition sites and mates, because both sexes were trapped, and females were in various stages of ovarian development (Schlein et al., 1989).

Ammonia has been reported to attract *Anopheles gambiae s.s.* at relatively high concentrations including 0.1–13.4 M (Braks et al., 2001) and 0.25–25% (Meijerink et al., 2001), and *Aedes aegypti* at much lower concentrations of 17 ppb to 17 ppm when presented with lactic acid (Geier et al., 1999). Conversely, although ammonia is a constituent of bird feces (Witter, 1991; Robacker et al., 2000), *C. quinquefasciatus* were shown to be activated by but not attracted to ammonia at concentrations spanning several orders of magnitude.

Cork and Park (1996) fractionated the volatile components of human sweat, and found L-lactic acid to be a major acidic component, with 1-octen-3-ol and decanal found in the non-acidic fraction. In the study reported here, acidifying aqueous slurries of fresh chicken feces improved mosquito attraction compared to the unacidified slurry at pH 7.3. Furthermore, the chemical profile of the headspace of the acidified slurry differed greatly from that of unaltered slurry, suggesting that the acidic conditions released volatile components that were present in the feces as nonvolatile conjugates, for example as Schiff bases (O'Brien et al., 2005). Steam distillation of slurries of acidified chicken feces appeared to eliminate attraction, because neither the steam distillate nor the residue remaining after distillation were attractive, suggesting that the active chemicals in the acidified solution may have been degraded by heat and/or acid during the distillation.

A number of compounds from both the acidified and unaltered chicken feces elicited antennal responses from female mosquitoes. We focused our attention on compounds from the acidified chicken

feces slurry because of its higher level of activity in the olfactometer versus the untreated slurry. Of the 10 compounds identified from acidified chicken feces out of the 18 that elicited the largest antennal responses, 8 had not previously been associated with mosquito olfaction. Location of these compounds in extracts was aided by use of multiple antennae in GC-EAD analyses, a method that has been previously documented as a way to improve the signal-to-noise ratio in GC-EAD studies with moth antennae (Moore, 1981; Park and Baker, 2002), but which has not been reported previously with mosquitoes. The compounds eliciting responses included (*E*)-2-decenal, undecanal, dodecanal, tetradecanal, pentadecanal, hexadecanal, heptadecanal, and octadecanal, and eight other EAD-active compounds that have not yet been identified (Fig. 3b). Some of these aldehydes (Table 2) have been identified in human skin odor (Bernier et al., 2000; Curran et al., 2007), but have not been targeted for mosquito behavioral or electrophysiological studies. The four positive controls (nonanal, 4-ethylphenol, indole, and 2-tridecanone) were chosen based on the antennal responses elicited by these compounds from female *C. quinquefasciatus* antennae in a study of oviposition attractants (Du and Millar, 1999). Two of the compounds listed by Du and Millar (1999), nonanal and 4-ethylphenol, also occurred in odors of acidified chicken droppings and elicited strong responses from antennae of females that had been conditioned for host seeking. The fifth control compound, 1-octanol, had not previously been tested in mosquito EAG studies, but it elicited a strong response from *C. quinquefasciatus* antennae.

The role of aldehydes as semiochemical cues appears to vary with mosquito and host species. For example, antennae of host-seeking *C. quinquefasciatus* females responded to benzaldehyde, heptenal, propanal, and nonanal, and females were attracted to the latter three compounds (Puri et al., 2006). Additionally, aldehyde components of waterbuck, ox, and buffalo odor elicited antennal responses from tsetse flies, and blends containing some of those aldehydes attracted *Glossina morsitans morsitans* (Gikonyo et al., 2002, 2003). In contrast, aldehydes in the feathers of auklets are apparently repellent to mosquitoes (Douglas et al., 2005).

In summary, we have shown that *C. quinquefasciatus* females were attracted to chicken feces. Using four mosquito antennae in parallel, we identified eight aldehydes that elicited strong responses from antennae of females to odors of acidified chicken feces. Strong and reproducible antennal responses were elicited by eight other compounds and these have not yet been identified. These results suggest host feces as a new avenue for investigations of the semiochemical basis of host attraction by host-seeking mosquitoes of medical and veterinary importance.

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